

# Prolonged survival of *Neisseria gonorrhoeae* in a new liquid medium

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## Summary

Despite substantial improvements in solid culture media for gonococci and the development of liquid media for the short-term growth of the organism, we know of no study of the long-term survival of *Neisseria gonorrhoeae* in liquid medium. A new liquid medium for *N. gonorrhoeae* formulated in this laboratory is simple, efficient, and promising. It will support growth and sustain inoculated colony types for periods of up to 3 weeks. Moreover, it has been used successfully for the primary isolation of gonococci from patients. From the evidence at present available, we believe that this medium will find a useful place in gonococcal research.

## Introduction

*N. gonorrhoeae* is a fastidious organism which for many years proved difficult to grow and to maintain in a viable state. One of the major advances in gonococcal research has been the development of well-defined and consistently successful media.

The recognition of the five main colony types by Kellogg, Peacock, Deacon, Brown, and Pirkle (1963) and Jephcott and Reyn (1971) amply demonstrated the value of modern media in the study of gonococci. However, it is generally true to say that liquid media and even diphasic media have been consistently less satisfactory than solid media, especially for the primary isolation of 'wild' strains of gonococci, and the main interest in the development of liquid media has been for the bulk growth of gonococci in order to study their antigens by Gerhardt and Heden (1960), Kenny, Ashton, Diena, and Greenberg (1976) and Chan, Wiseman, and Caird (1975).

## Material and methods

### Strains

In all 106 strains were studied. Fifty, freshly isolated from patients attending the Sheffield Special Clinic, were supplied by Dr. A. E. Jephcott; a further 31 strains were

isolated in this laboratory from clinic patients and the final 25 were earlier isolates which had been stored in liquid nitrogen for periods from 30 to 60 weeks.

### Media

The solid medium used in this study was DIFCO GC medium plus 2 per cent. Defined Supplement (DS) (L-glutamine 1.0 g., Dextrose 40.0 g., Ferric nitrate 0.05 g., and Cocarboxylase 0.02 mg. in 100 ml. distilled water). For the isolation of gonococci from clinical specimens, Vancomycin (3 µg./ml.), Trimethoprim (8 µg./ml.), and Colistin (7.5 µg./ml.) were added.

The liquid medium used is based on Difco GC-base medium and has the following composition:

Proteose peptone (DIFCO 3)	..	15.0 g.
Sodium chloride (AnalaR)	..	5.0 g.
Dipotassium phosphate (AnalaR)	..	4.0 g.
Potassium di-hydrogen phosphate (AnalaR)	..	1.0 g.
*Corn or soluble starch (AnalaR)	..	1.0 g.
Sodium bicarbonate (AnalaR)	..	0.15 g.
Glucose (AnalaR)	..	5.0 g.
Distilled water	..	1,000 c.c.
pH adjusted to 7.4 (7.2 — 7.4) with N/NaOH		

This basic liquid medium was sterilized by autoclaving at 10 lb. for 15 min. After sterilization 2 per cent. sterile DS as described above was added. The basic medium was then compared with the medium plus supplement.

Both media were distributed in 15 ml. volumes in universal containers and 10 ml volumes in cotton-wool plugged 6-in. ×  $\frac{5}{8}$ -in. test tubes. For convenience the medium was referred to as ANM.

### Culture

Inocula were prepared from both freshly isolated strains and established laboratory strains in the following way. After subculture on to solid GC medium plus 2 per cent. DS and incubation at 35 to 36°C. for 18 to 20 hrs, growth from surface cultures was scraped off and suspended in phosphate buffered saline (PBS) at pH 7.4 to give a viable count of  $10^8$  organisms/ml., and 0.1 ml. of such a suspension was used to inoculate two parallel series of 15 ml. volumes of ANM, one with and one without DS. Liquid cultures were then incubated (a) in an atmosphere of air and (b) in an atmosphere of enhanced (10 per cent.) carbon dioxide.

### Measurement of growth

The number of viable organisms was determined by the method of Miles, Misra, and Irwin (1938), using DIFCO

\*Subsequently soluble starch was found superior.

GC base plus 2 per cent. DS. Changes in the optical density of the liquid medium during growth were also recorded by serial Nephelometer readings using an EEL Nephelometer. The calibration of the Nephelometer was checked and confirmed from time to time by carrying out viable counts.

Every 24 hrs the liquid cultures were subcultured on to solid GC medium to observe the colonial types present, and a viable count was carried out by taking 0.1 ml. into 9.9 ml. PBS. These observations were also checked by reading the optical density using the Nephelometer. In a further experiment the minimum inoculum needed to ensure growth in ANM was estimated by using serial dilutions of inoculum and subsequent observation on the viable counts of the resulting growth.

#### *Isolation of N. gonorrhoeae from patients*

When using ANM for the isolation of 'wild' strains from patients, antibacterial agents were added in the same way as for solid media. Standard DIFCO GC agar plates and bottles of ANM were inoculated with urethral discharge using a sterile wire loop. The inoculated plates and ANM containers were incubated at 35 to 36°C in an atmosphere of enhanced humidity and 10 per cent. carbon dioxide, and examined for growth after 24, 48, and 72 hrs. In ANM, growth was seen as an increase in turbidity and the nature of the growth was confirmed by subculturing a loopful of the broth on to solid GC medium. The identity of isolates was confirmed by Gram staining, oxidase reaction, sugar fermentation, and the fluorescent antibody tests using fluorescein conjugated gonococcal antiglobulin from Difco Laboratories and the method recommended by the makers. Cultures were examined for purity and colonial type of gonococci. Negative results were recorded when no growth was observed at the end of 7 days' incubation.

## Results

Comparison of the average of Nephelometer readings (increase in density) and viable counts obtained with 24 strains of gonococci gave identical results, which are shown in Figs 1 and 2, in which the following comparisons were made:

(1) ANM alone compared with ANM plus DS, both being incubated in air.

(2) ANM alone compared with ANM plus DS, both being incubated in an atmosphere of 10 per cent. carbon dioxide.

The Figures show that the presence of DS results in more rapid growth, but in consequence the survival time of the organism is reduced. In addition the presence of DS makes the culture more sensitive to the presence of CO<sub>2</sub>.

Fig. 3 gives the comparison of growth in ANM with corn starch and with soluble starch. From these findings it is clear that soluble starch favours the growth of *N. gonorrhoeae*.

Table I shows the effect of DS on the relative generation times of gonococci grown in media containing soluble starch or corn starch. In the presence

of DS the generation time is shortened, even when the organism is incubated in air.

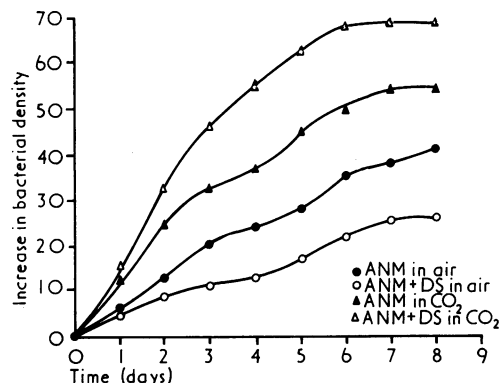


FIG. 1 Effect of DS and carbon dioxide on the growth (density) of *N. gonorrhoeae* in ANM

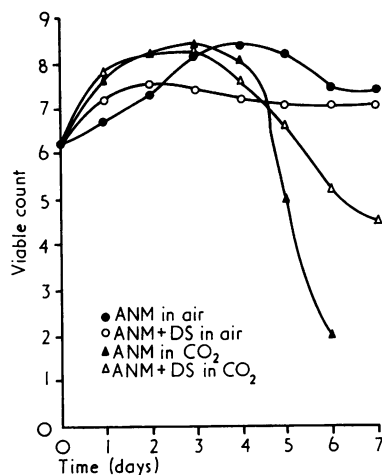


FIG. 2 Effect of DS and carbon dioxide on the growth (viable count) of *N. gonorrhoeae* in ANM

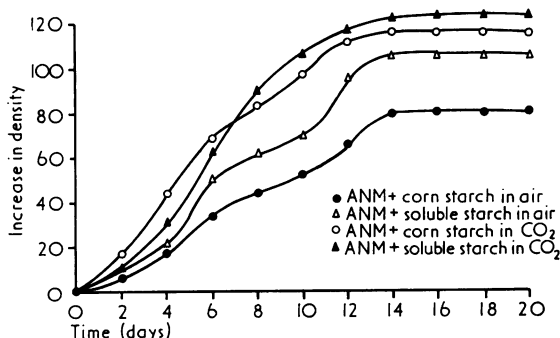


FIG. 3 Effect of corn starch and soluble starch on the growth (density) of *N. gonorrhoeae* in ANM

TABLE I *Generation time (hrs) of gonococci in ANM incubated in air (average values taken from 24 observations)*

ANM containing corn starch		ANM containing soluble starch	
ANM	ANM + 2 per cent. DS	ANM	ANM + 2 per cent. DS
4.80	1.22	4.40	0.767 (46.08 min)

In a series of experiments to determine the minimum inoculum for growth in ANM, the average value was found to be about 50 to 500 organisms/ml. of the medium; for example, when using 10 ml. ANM, 5,000 organisms were required to give a consistent increase in turbidity (as recorded by Nephelometer) in 24 hrs.

Table II illustrates the comparative survival times of 106 strains of gonococci in ANM with and without DS. It was interesting to note that strains remained in colony type 1 for most of the time they survived. A preponderance of type 4 colonies was observed in subcultures only on the final 1 to 3 days before the cultures became sterile. The minimum survival time of the strains in ANM + DS was 6 days, the maximum was 49 days, and most of the strains survived for 12 days, while in ANM without DS, the minimum survival time was 9 days, the maximum was 60 days, and the majority of the strains survived for 15 days.

TABLE II *Duration of survival (as judged by growth on subculture) of 106 strains of gonococci incubated at 36°C. in (a) ANM + 2 per cent. DS and (b) ANM (Air)*

Time (days)	<sup>a</sup> ANM + 2 per cent. DS		<sup>b</sup> ANM	
	Total no. surviving	Percentage surviving	Total no. surviving	Percentage surviving
6	106	100	106	100
7	104	98.11	106	100
11	69	65.09	102	96.22
14	25	23.58	78	73.58
21	9	8.49	33	31.13
28	7	6.60	20	18.86
35	1	0.94	16	15.09
49	1	0.94	6	5.66
56	—	—	4	3.77
63	—	—	—	—

Table III compares ANM and solid media for the isolation of gonococci from clinical material. The rate of isolation on solid medium was 80.35 per cent., while the rate of isolation in ANM was 91.07 per cent. The isolates survived for from 2 to 35 days in the liquid medium, the majority for 11 days. Shorter survival times were all observed when the cultures were overgrown by *Candida albicans*.

TABLE III *Comparison of solid and liquid media for the isolation of strains of N. gonorrhoeae from patients*

Total no. of patients	Solid Medium				ANM			
	Positive	Negative	Contaminated	Per cent. Positive	Positive	Negative	Contaminated	Per cent. Positive
56	45	11	13	80.35	51	5	21	91.07

## Discussion

Our observations on the liquid media show that:

- (1) In the presence of DS, gonococci multiply rapidly, the generation time being considerably reduced, and at the same time the organism survives for a shorter period than comparable strains in ANM without DS. An atmosphere of increased carbon dioxide affects the overall growth to a slight extent, but as might be expected is not so important in liquid as on solid media.
- (2) Gonococci survive much longer in liquid than on solid media.
- (3) In liquid medium the long survival of virulent types will provide an opportunity to study the accumulation of toxic or other breakdown products and should facilitate studies on the release of endotoxin.
- (4) The shelf life of the medium in our laboratory is about 6 months.

The role of ANM in the diagnostic field seems very promising, particularly as a selective transport/growth medium. It may also prove of value in the study of the MICs of various antibacterials especially as it permits growth of recent isolates without the need for adaptation. We believe that the use of this liquid medium will be a valuable addition to our research methods.

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